Identification, Phylogenetic Analysis, and Biological Characterization of *Serratia marcescens* Strains Causing Cucurbit Yellow Vine Disease

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ABSTRACT

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A serious vine decline of cucurbits known as cucurbit yellow vine disease (CYVD) is caused by rod-shaped bacteria that colonize the phloem elements. Sequence analysis of a CYVD-specific polymerase chain reaction (PCR)-amplified 16S rDNA product showed the microbe to be a γ-proteobacterium related to the genus *Serratia.* To identify and characterize the bacteria, one strain each from watermelon and zucchini and several noncucurbit-derived reference strains were subjected to sequence analysis and biological function assays. Taxonomic and phylogenetic placement was investigated by analysis of the *groE* and 16S rDNA regions, which were amplified by PCR and directly sequenced. For comparison, eight other bacterial strains identified by others as *Serratia* spp. also were sequenced. These sequences clearly identified the CYVD strains as *Serratia marcescens*. However, evaluation of metabolic and biochemical features revealed that cucurbit-derived strains of *S. marcescens* differ substantially from strains of the same species isolated from other environmental niches. Cucurbit strains formed a distinct cluster, separate from other strains, when their fatty acid methyl ester profiles were analyzed. In substrate utilization assays (BIOLOG, Vitek, and API 20E), the CYVD strains lacked a number of metabolic functions characteristic for *S. marcescens*, failing to catabolize 25 to 30 compounds that were utilized by *S. marcescens* reference strains. These biological differences may reflect gene loss or repression that occurred as the bacterium adapted to life as an intracellular parasite and plant pathogen.

Additional keywords: cantaloupe, *Enterobacteriaceae*, niche-adaptation, opportunistic pathogen, phloem-resident bacterium, squash, watermelon, zucchini.

Cucurbit yellow vine disease (CYVD) first was observed in Oklahoma in 1988 in pumpkin and squash (8,10). Exhibiting characteristic symptoms of yellowing foliage, wilting, and phloem discoloration, this disease has since caused severe losses also in cantaloupe and watermelon crops. Disease incidence has varied annually from small, isolated outbreaks to complete crop loss, especially in early-planted fields (8). Originally thought confined to Oklahoma and central Texas, CYVD has since been confirmed in Arkansas (J. C. Correll, *personal communication*), Tennessee (6), Massachusetts (43), Kansas (N. A. Tisserat, J. Fletcher, and B. D. Bruton, *unpublished data*), Colorado (B. D. Bruton, *unpublished data*), and Nebraska (R. M. Harveson and B. D. Bruton, *unpublished data*).

Disease symptoms consistently are associated with the presence of rod-shaped bacteria in phloem sieve elements, detected using transmission electron microscopy (8). Avila et al. (3) amplified a nearly full-length 16S rDNA fragment from infected tissue. The nucleotide sequence of this fragment suggested that the organism was a member of the γ-3 proteobacteria, with the nearest apparent relative being in the genus *Serratia.* From that sequence, Avila et al. (3) designed CYVD-targeted 16S rDNA primers, and Melcher et al. (27) later developed additional primers with greater specificity for CYVD, for use in polymerase chain reaction (PCR). PCR screening of CYVD-infected and healthy cucurbit plants established a consistent association between the amplification of the specific PCR products and CYVD symptoms. The bacterium was isolated from diseased plant phloem and cultivated on artificial medium, and completion of Koch's postulates demonstrated that this microbe was indeed the etiological agent of CYVD (7–9). Furthermore, transmission of the CYVD bacterium from plant to plant was accomplished both mechanically, by needle inoculation of seedling cotyledons, and via the squash bug, *Anasa tristis*, a cucurbit insect pest not previously known to transmit any plant pathogen (4,5,7,9).

The goals of this work were to identify and partially characterize the CYVD causal bacterium. Two CYVD strains and selected reference strains were evaluated phylogenetically by nucleotide sequencing of the 16S rDNA and *groE* regions of the chromosome. Analysis of 16S rDNA sequences is one of the most useful and widely accepted means for assessment of the natural and taxonomic relationships of microorganisms (18,19,42). However, because this gene is so highly conserved among species of the family *Enterobacteriaceae*, of which the genus *Serratia* is a member, attempts by others to construct molecular phylogenetic trees for this bacterial family based on 16S rDNA have been of limited success. Analyses of *groE* sequences, however, have been useful in the classification of closely related bacterial species, especially those in the family *Enterobacteriaceae* whose 16S rDNA sequences are too highly conserved to be effective for distinction (16). The *groE* operon, encoding the chaperonin stress proteins GroES and GroEL, is highly conserved in the bacterial world and is essential for viability (20,28). The CYVD and reference bacterial strains also were characterized biologically, using fatty acid profiling and substrate utilization tests (BIOLOG, Vitek, and API 20E).

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Our results show that the CYVD bacterium is, indeed, a member of the genus *Serratia*, and, in fact, that it is *Serratia marcescens*. Further, they demonstrate that the CYVD strains differ significantly in their biological functions and characteristics from other selected noncucurbit strains, which had been isolated from soil, plant interiors (nonpathogenic associations), and humans. A preliminary abstract has been published (30).

MATERIALS AND METHODS

Bacterial strains, growth conditions, and preliminary characterization. Two CYVD-derived bacterial strains, W01-A (watermelon) and Z01-A (zucchini), initially were cultivated on purple agar (Sigma-Aldrich, St. Louis) from phloem tissue excised from the crowns of infected plants, triply cloned to assure homogeneity, and maintained in frozen aliquots in 50% glycerol at –80°C as described (7,9). Their identity was confirmed by the production of an amplicon of expected size using CYVD-specific primers in PCR (27) and by sequencing of the PCR product (Table 1). The type strain of *S. marcescens* (ATCC no. RF4738) and eight additional *Serratia* strains, originating from noncucurbit niches, were obtained from other investigators (Table 1). Bacteria were grown in Luria-Bertani (LB) broth (31) for 8 to 12 h with shaking at 28°C. Gram status of the cell walls was determined by the KOH test, and the ability to grow under anaerobic conditions was tested using Hugh and Leifson's medium (O-F) sealed with Vaseline; both of these experiments were done as described by Schaad (32).

DNA isolation. DNA was extracted using a modified version of the hexadecyl trimethyl ammonium bromide (CTAB) method (2). Briefly, 3 ml of log-phase cells were centrifuged for 5 min at 10,000 × *g* and 4°C, supernatant was removed, and cells were resuspended in 200 µl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The mixture was incubated for 30 min at 60°C; then, 100 µl of 5 M NaCl and 80 µl of CTAB solution (10% CTAB in 0.7 M NaCl) were added and mixed, and the suspension was incubated at 60°C for 10 min. The solution was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), followed by DNA-precipitation and a wash with isopropanol and 70% ethanol, respectively.

PCR. Amplification and sequencing of the *groE* region of the genome was performed with three primer sets: A1/B1, A2/B2, and A3/B3, as described by Harada and Ishikawa (18). Each reaction included 30 ng of template DNA in a volume of 25 µl containing 3.5 mM $MgCl_2$, 0.05 mM each dCTP, dATP, dTTP, and dGTP, 200 pM of each primer (A1/B1, A2/B2, and A3/B3), and 1.5 units of *Taq* polymerase (Promega Corp., Madison, WI). The DNA was amplified under the following conditions: 3 min at 95°C; 35 cycles of 30 s at 94°C, 60 s at 55°C, and 60 s at 70°C; and final elongation at 72°C for 10 min.

Amplification and sequencing of 16S rDNA was achieved using universal primers fD1 and rP2 (21) and primers α (5′-CTGCTGCCTCCCGT-3′), β (5′-CTACTCGGGTATCTAATC-3′), and γ (5′-AGGGTTGCGCTCGTTG-3′) (C. Sproer, *personal communication*), amplifying position numbers 29 to 1,492, inclusive of the M59160 sequence for *S. marcescens* strain ATCC13880, as determined by Woese (45). Each reaction contained 30 ng of DNA, isolated from the organisms as described previously, in a total volume of 25 µl containing 3.5 mM $MgCl_2$, 0.05 mM each dCTP, dATP, dTTP, and dGTP, 200 pM of each primer, and 1.5 units of *Taq* polymerase. Amplification was for 95°C for 3 min; 35 cycles of 60 s at 92°C, 45 s at 47°C, and 90 s at 72°C; followed by 10 min at 72°C.

For analysis, PCR products were electrophoretically separated in 1.5% MetaPhor agarose (FMC BioProducts, Rockville, MD) containing ethidium bromide at 50 µg/liter, and band sizes were estimated by comparison with 1-kb Plus DNA Ladder molecular weight markers (Gibco-BRL, Rockville, MD). The resultant PCR products were excised from the gel and prepared for direct se-

TABLE 1. Bacterial strains characterized by sequence analysis and sources of sequences used in phylogenetic tree generation in this study

Bacterial identity	Strain	Source[b]	GenBank accession number[a]	
			16S rDNA	*GroE*
Serratia spp. used				
S. marcescens	W01-A	CYVD watermelon (Texas)	AJ296309	AJ297252
S. marcescens	Z01-A	CYVD zucchini squash (Oklahoma)	AJ296310	AJ297253
S. marcescens	90-166[c]	Cotton root (Alabama)	AJ296306	AJ297249
S. marcescens	98A-742[c]	Soil treated with DiTera (Florida)	AJ296307	AJ297250
S. marcescens	CP01(4)CU[c]	Rhizosphere, golf course (Florida)	AJ296308	AJ297251
S. marcescens	JM-965[c]	Cotton stems (Florida)	AJ302154	AJ302157
S. plymuthica	JM-983[c]	Cotton roots (Florida)	AJ302155	AJ302158
S. marcescens	HO1-A[d]	Human, clinical (Oklahoma)	AJ297946	AJ297956
S. marcescens	H02-A[d]	Human, clinical (Oklahoma)	AJ297950	AJ297957
Serratia sp.	IRBG 501[e]	Rice (Philippines)	AJ302156	AJ302159
Serratia sequences[f]				
S. marcescens	JCM 1239	…	…	AB008145
S. marcescens	DSM 30121	…	AJ233431	…
S. rubidaea	JCM 1240	…	…	AB008143
S. rubidaea	DSM 4480	…	AJ233436	…
S. ficaria	JCM 1241	…	…	AB008144
S. ficaria	DSM 4569	…	AJ233428	…
Non-*Serratia* sequences[f]				
Enterobacter asburiae	JCM 6051	…	AB004744	AB008137
Klebsiella planticola	JCM 7251	…	…	AB008148
K. planticola	ATCC 33531T	…	X93216	…
Erwinia carotovora	IAM 12633	…	…	AB008152
E. carotovora	DSM 30168	…	Z96090	…
Sitophilus oryzae endophyte	…	…	AF005235	AF005236

[a] Database reference sequences obtained from GenBank. Accession numbers beginning with "AB" are from the DNA databank of Japan, while those beginning with "AJ" are from the EMBL sequence database.
[b] CYVD = cucurbit yellow vine disease.
[c] Provided by J. Kloepper, University of Alabama, Auburn, AL; who identified the bacteria by fatty acid methyl ester analysis.
[d] Provided by D. Adamson, Medical Arts Laboratory, Oklahoma City, OK; who identified the bacteria by substrate utilization using API 20E.
[e] Provided by J. K. Ladha, International Rice Research Institute, Los Baños, Philippines; who identified the bacteria by 16S rDNA sequencing (16).
[f] Sequences from databases used as reference sequences in this study.

quencing using a QIAquick gel extraction kit (Qiagen Inc., Valencia, CA). The gel-purified DNAs were quantified by electrophoresis and compared with herring sperm DNA of known quantity. Automated sequencing was performed by the Oklahoma State University Recombinant DNA/Protein Resource Facility, using dye-terminated thermal cycle sequencing and an Applied Biosystems/Perkin-Elmer 373 sequencer (Perkin-Elmer Inc., Wellesley, MA).

Sequence editing and phylogenetic tree construction. Resulting sequences were aligned and edited using BioEdit software (version 4.7.3; T. Hall, North Carolina State University). Phylogenetic trees based on *groE* and 16S rDNA sequences were compiled using PHYLIP programs (13). The 16S rDNA and *groE* sequences of six other plant- or insect-related members of the family *Enterobacteriaceae* were obtained from GenBank (Table 1). Distance matrices were calculated with DNADIST and used to construct trees by neighbor joining as implemented in NEIGHBOR. The phylogenetic trees were displayed using TreeView (29). Alignments were bootstrapped 1,000 times using SEQBOOT, and bootstrap values were added to the internal branches of the distance trees. Branches with bootstrap values less than 500 were collapsed.

Fatty acid and substrate utilization tests. Triply cloned cultures of each bacterium were streaked onto purple agar (Difco Laboratories, Detroit), grown at 28°C, and provided to E. Dickstein, University of Florida, Gainesville, for analyses of fatty acid methyl ester (FAME; MIDI, Inc., Newark, DE) and substrate utilization (BIOLOG, Inc., Hayward, CA) profiles, which were performed following manufacturers' instructions. The BIOLOG tests were done using substrate plates designed for gram-negative bacteria. FAME testing was repeated a total of three times, and BIOLOG twice. The Vitek and API 20E (BioMérieux, Hazlewood, MO) automated bacterial identification assays, which included substrate utilization and antibiotic sensitivity tests and were analyzed by comparisons with a database composed of medically important strains, were performed once each by D. Adamson, Medical Arts Laboratories, Oklahoma City, OK, following manufacturers' instructions.

RESULTS

Preliminary characterization. When streaked onto agar-solidified LB medium, cucurbit-derived bacterial strains all produced smooth, circular, entire, convex, nonpigmented colonies within 24 h. All the tested bacterial strains were gram negative, as indicated by the formation of dense, slimy threads when loopfuls of bacteria were stirred into KOH solution. After being stabbed into O-F medium, all strains grew and produced acidic byproducts, as indicated by a color change in the acid-base indicator, whether covered or not covered with sterile Vaseline (Table 2). These data demonstrate that all of the tested strains are facultative anaerobes.

Nucleic acid sequencing. Approximately 1.3 kb of *groE* (a fragment containing approximately 80% of the *groES* gene and 60% of the *groEL* gene) and 1.4 kb of 16S rDNA (approximately 91% of the gene) homologues were sequenced for the two CYVD and eight reference strains. Sequences were subjected to multiple sequence alignments. To obtain reliable alignments for the *groE* sequences, the intergenic region between *groES* and *groEL* was excluded. The *groE* sequences of the two CYVD strains and six of the other eight strains were highly similar to each other, as reflected in pairwise distance values averaging 0.01 ± 0.005 nucleotide substitutions per site for the sequences from these eight strains (two CYVD and six others). In contrast, the *groE* sequences of the remaining two reference strains, JM-965 and JM-983, both of which are endophytes of cotton, were significantly more distant from the cluster of eight and from each other, with mean distances of 0.10 ± 0.005 for JM-965 and 0.045 ± 0.004 for JM-983. For 16S rDNA sequences, the mean pairwise distance was 0.002 ± 0.0012 within the eight closely related strains, a value five times lower than that obtained for the *groE* set. For the two more distantly related strains, JM-965 and JM-983, values were 0.023 ± 0.0009 and 0.034 ± 0.0125, respectively.

Phylogenetic tree development. Parsimony analysis of both sequence sets failed to produce phylogenetic trees with reliable branches, due to the small number of phylogenetically informative positions in the data sets. Positions with substitutions in only one lineage contribute to phylogenetic distance; therefore, distances (number of substitutions per site) between all pairs of sequences in each set were calculated by DNADIST.

Phylogenetic trees were generated from the distance data using the PHYLIP program NEIGHBOR (Figs. 1 and 2). Homologous sequences from other known members of the family *Enterobacteriaceae* having plant or insect associations were included in the phylogenetic analysis for comparison (Table 1). The bacterial symbiont of *Sitophilus oryzae*, an enterobacterium closely related to *Serratia* spp. for which both 16S rDNA and *groE* sequences were available, was chosen as the outgroup. For 16S rDNA data,

TABLE 2. Partial substrate utilization profiles of the cucurbit bacterial strains W01-A and Z01-A, the cotton endophyte *Serratia marcescens* 90-166, the clinical strain *S. marcescens* H02-A, and a reference strain of *S. marcescens*[a]

Test	W01-A	Z01-A	90-166	H02-A	*Sm*[b]
BIOLOG					
α-D-Lactose	0	0	0	+	+
α-Hydroxybutyric acid	0	0	±	+	+
α-Ketoglutaric acid	±	0	+	+	+
β-Hydroxybutyrate	0	0	+	+	+
Adonitol	0	0	+	+	+
Alaninamide	0	0	+	+	+
Bromosuccinic acid	±	0	+	+	+
Cellobiose	0	0	±	+	±
DL-Lactic acid	0	0	+	+	+
D-Alanine	0	0	+	+	+
D-Arabitol	0	0	+	+	+
D-Mellobiose	±	0	+	+	+
D-Serine	0	0	+	+	+
D-Sorbitol	±	0	±	+	+
Gentobiose	0	0	+	+	+
Glucuronamide	±	0	+	+	+
Hydroxy-L-proline	0	0	+	+	+
i-Erythritol	0	0	+	+	±
L-Alanine	0	0	+	+	+
L-Ananyl glycine	0	0	+	+	+
L-Arabinose	0	0	+	+	+
L-Aspartic acid	0	0	+	+	+
L-Fucose	0	0	+	+	+
L-Ornithine	0	0	±	+	+
L-Phenylalanine	0	0	±	±	+
m-Inositol	0	0	+	+	+
N-Acetyl-D-galactosamine	0	0	+	+	+
Putrescine	0	0	+	+	+
Succinamic acid	±	0	+	±	±
Thymidine	0	0	+	+	+
Vitek					
Adonitol fermentation	0	0	+	ND	+
Coumarin	0	0	+	ND	+
Esculin hydrolysis	+	+	0	ND	±
Inositol fermentation	0	0	+	ND	+
Lysine	0	0	+	ND	+
Maltose fermentation	0	0	+	ND	0
Ornithine	0	0	+	ND	+

[a] BIOLOG and Vitek data are presented only for the reactions in which cucurbit yellow vine disease (CYVD) strains differed unequivocally from those of reference strains. For example, in BIOLOG, utilization profiles of the CYVD strains were indistinguishable from those of reference strains for 65 of the 95 substrates. Symbols: 0 = no activity, + = activity; ± = questionable, and ND = not done.

[b] BIOLOG and Vitek database information for a reference strain of *S. marcescens* is included for comparison; these strains were not tested in our assays.

branching patterns varied considerably, depending on which distantly related sequence was included as outgroup and on which other members of *Enterobacteriaceae* were included in the data set. However, the relative 16S-based placement of the bacteria used in our study remained consistent despite these variables, and clearly identified the CYVD strains as *S. marcescens.*

The *groE*-based phylogenetic tree also placed the two cucurbit bacterial strains among known strains of *S. marcescens.* In contrast to the low reliability of the 16S rDNA trees, branching patterns generated for species within the family *Enterobacteriaceae* using *groE* data were similar to each other. Because of this consistency, the confidence level associated with phylogenetic relationship prediction using the *groE* sequence data is higher than that using the 16S rDNA data. In addition, the correspondingly high bootstrap values result in significant branch points in the *groE*-based phylogenetic tree that are not present in the 16S tree. Harada et al. (18), who characterized a number of members of the family *Enterobacteriaceae* on the basis of their *groE* sequences, omitted the spacer region between the *groES* and *groEL* genes to obtain a reliable alignment for phylogenetic comparison. In our study, including or deleting this spacer region made no difference in the resulting phylogenetic trees, even though there were clear base differences in this segment.

In addition to placing the CYVD strains W01-A and Z01-A close to known *S. marcescens* strains DSM 30121 and JCM 1239, both the 16S-based and the *groE*-based trees show their close relationship to the human clinical strains H01-A and H02-A. Also included in this closely related grouping are soil strains CP01(4)CU and 98A-742, and plant endophytes 90-166 (cotton) and IRBG 501 (rice).

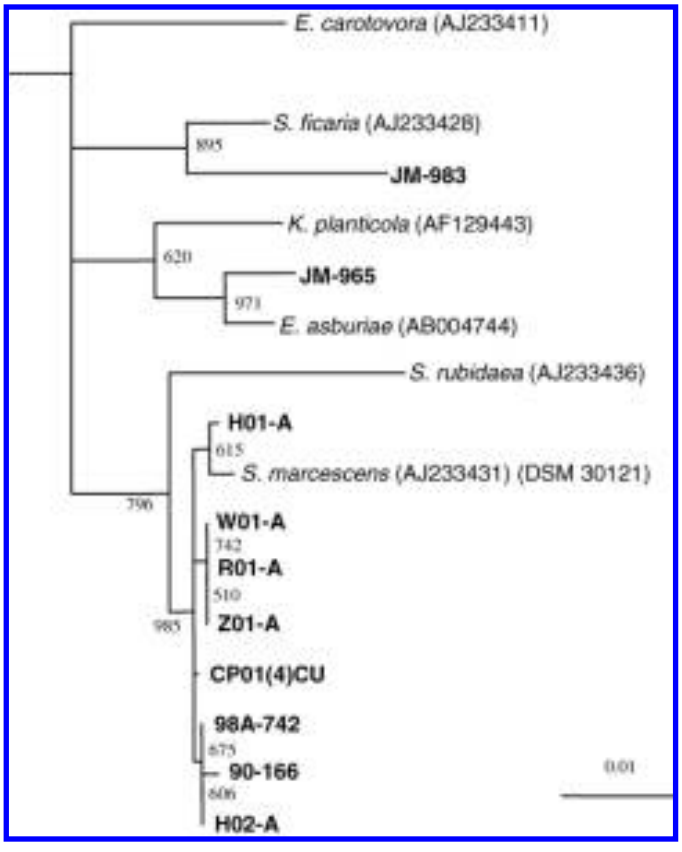

Fig. 1. Phylogenetic distance tree compiled from 16S rDNA sequence data using programs DNADIST and NEIGHBOR, with the endosymbiont of *Sitophilus oryzae* as outgroup. Branches with bootstrap values less than 500 were collapsed, and two branches (bootstrap values 510 and 606) were of relative lengths insufficient for resolution at the scale of this figure. Strains indicated in bold font were used in this study; the remainder are database reference strains. RO1-A is the rice strain IRBG 501.

As mentioned previously, strains JM-983 and JM-965, isolated from cotton roots and stems, respectively, were outside the *S. marcescens* cluster. A BLASTn search initiated with the *groE* sequence from JM-983 yielded *S. marcescens* JCM 1239 as the best fit, with 1,239 of 1,292 nucleotides (nt) identical. BLASTn analysis of the 16S rDNA sequence identified *S. grimesii* DSM 30063 as the most similar to this strain, with 1,402 of 1,406 nt identical. *S. grimesii* was not included in our phylogenetic trees because *gro*E sequence data was unavailable for this organism. Of all the strains compared, JM-965, a cotton stem endophyte, is the least related to the others. Here again, *gro*E and 16S sequence database searches yielded somewhat different results; *Enterobacter asburiae* JCM 6051 was the species having greatest similarity to the JM-965 *gro*E sequence by BLASTn search (1,233 of 1,282 nt), whereas *E. cloacae* (1,402 of 1,406 nt) was the most similar 16S rDNA sequence available in the databases.

Fatty acid analysis. Although comparisons of CYVD bacterial fatty acid profiles with those of known bacteria in the MIDI database revealed "closest match" genera and species, the similarity indices of these matches were very low. However, all of the closest match species were in the family *Enterobacteriaceae*, and the cucurbit strains possessed a 14:0 3-hydroxy fatty acid that is characteristic of this bacterial family.

The MIDI program (12) was used to generate a principle component analysis comparing the fatty acid makeup of the CYVD bacterial strains with those of specific reference and database strains of *Serratia* spp. The two cucurbit strains, W01-A and Z01-A, clustered together, well separated from all known database *Serratia* spp. (Fig. 3). They also clearly were different from two strains, H02-A and 90-166, used as references in the current study. The relationship between any two strains was estimated by multiplying the difference between their *x*-axis positions by the distance between their *y*-axis positions. MIDI instructions suggest that values of ≥110 are indicative of separate species, while values of <60 suggest that two strains are in the same subspecies. Bacteria having values of <30 are recommended for assignment to the same strain.

In this analysis, the only within-species comparison of established strains is that of *S. marcescens* A versus *S. marcescens* B (the two groups of database *S. marcescens*, distinguishable from one another by their fatty acid profiles), which yielded a value of 6.2. To create a larger comparison group, the two analyses of the known human strain, H02-A, were added to those of *S. marcescens* A and B, yielding a range of scores from 4.2 to 7.8. Among all the between-species comparisons, six were in or below this range: *S. rubidaea* versus *S. marcescens* A, *S. rubidaea* versus *S. marcescens* B, *S. rubidaea* versus *S. odorifera*; *S. odorifera* versus *S. fonticola*, *S. odorifera* versus *S. grimesii*; and *S. liquifaciens* versus *S. marcescens* B. Five of the six involved *S. rubidaea* and *S. odorifera*, suggesting that these bacteria may be very similar. The bulk of the comparisons between species acknowledged to be different yielded values from 11.0 to 168. These values are, on average, considerably less than the MIDI standard of ≥110.

Clearly, the general MIDI standards are inadequate for *Serratia* spp., although the reason is not clear. Substituting the within-species MIDI range of 4.2 to 7.8, and between-species range of 11 to 168 (derived from the data for established species), W01-A and Z01-A were placed in the same species as *S. fonticola*, *S. plymuthica*, *S. odorifera* (except one of the three Z01-A replications), and 90-166B. This approach clearly separated the two cucurbit strains from *S. marcescens* A and B, *S. marcescens* H01-A, *S. rubidaea*, *S. liquifaciens*, *S. grimesii*, *S. odorifera* (one of the three Z01-A replications only), and 90-166A.

Substrate utilization and other biological features. Analyses based entirely (BIOLOG) or predominantly (Vitek and API 20E) on substrate utilization profiles revealed indistinguishable reaction patterns for the two CYVD strains, and showed that they were unequivocally distinct in these tests from the eight test reference

strains and from all known *Serratia* strains in the corresponding databases. For example, although W01-A and Z01-A had the same utilization reactions as the reference *S. marcescens* for 65 of the 95 substrates on the Biolog plates, they differed in their ability to metabolize the other 30 substrates (Table 2). Interestingly, in all but one case (esculin hydrolysis), the differences in BIOLOG substrate utilization reflected a lack, in the cucurbit strains, of functions present in the *S. marcescens* reference strains. Although BIOLOG data showed that CYVD strain W01-A had the highest similarity to *Aeromonas veronii* and that Z01-A had the highest similarity to *Vibrio cholerae*, the similarity rankings (30 and 40%, respectively) were too low to be considered reliable. In the Vitek analysis, the reaction profiles of the CYVD strains were so dissimilar from those of the databank species that the automated system found no species matches. In addition, DNAse activity, often considered diagnostic for *S. marcescens* (20), was not detected in the cucurbit strains (API-20, data not shown). In contrast to the somewhat contradictory results for strains W01-A and Z01-A, the endophytic reference strain, 90-166, was clearly identified as *S. marcescens* by both BIOLOG (91% similarity) and Vitek (99% similarity).

DISCUSSION

CYVD, a disease of significant economic importance in several regions of the United States, is caused by a cultivable, gram-negative, facultatively anaerobic phloem-resident bacterium. Preliminary sequence comparisons of 16S rDNA PCR amplified directly from infected plant tissue (3) suggested that it was a γ-proteobacterium in the family *Enterobacteriaceae*, related to *Serratia* spp. In the work reported here, rigorous analysis of sequences from both 16S rDNA and *groE* genomic regions of cultivated and triply cloned bacterial strains demonstrated that the CYVD bacterium is *S. marcescens*. The phylogenetic relationships among *S. marcescens* and other members of *Enterobacteriaceae* have been well documented (19,37,45).

The identity of the pathogen was unexpected because *S. marcescens* is not typically associated with plant disease and had not been known previously to colonize plant phloem. Capable of thriving in diverse habitats and ecological niches, *S. marcescens* has been isolated from soil, water, plants, food products, and in-

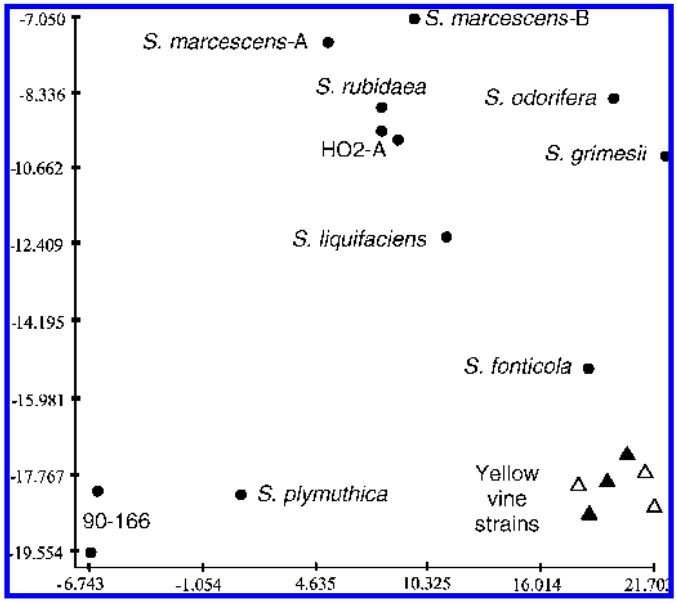

Fig. 3. Principle component analysis of fatty acid methyl ester profile data for cucurbit strains W01-A (black triangles) and Z01-A (white triangles), the human strain *Serratia marcescens* H02-A, the endophyte *S. marcescens* 90-166, and several *Serratia* reference strains.

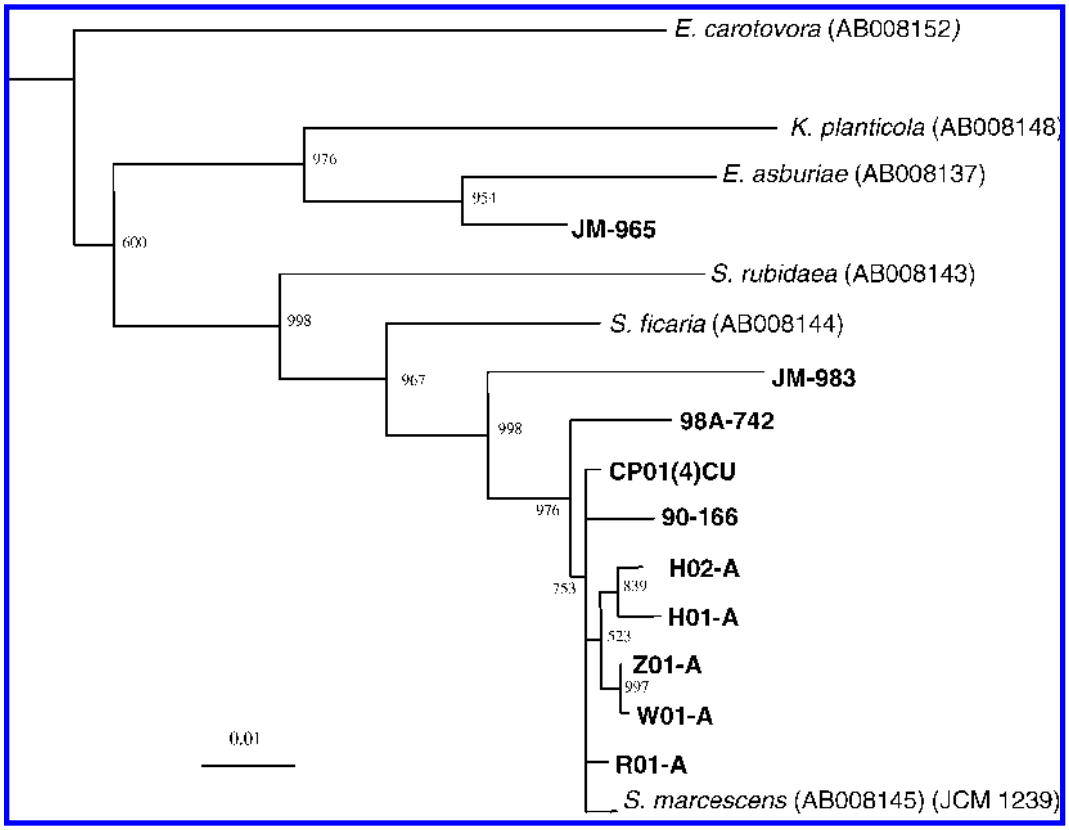

Fig. 2. Phylogenetic distance tree compiled from *groE* sequence data, using programs DNADIST and NEIGHBOR, with the endosymbiont of *Sitophilus oryzae* as outgroup. Branches with bootstrap values less than 500 were collapsed. Strains indicated in bold font were used in this study; the remainder are database reference strains. RO1-A is the rice strain IRBG 501.

fections of animals, including insects and humans (1,16,22,33, 36,38). Various plant-associated roles are filled by this species, including that of a plant-growth-promoting rhizobacterium (24, 25); an innocuous colonizer or endophyte of plants, including cotton and rice (39,41); and even a biocontrol agent capable of reducing or preventing infections by bacterial or fungal pathogens (25,46). Previous to this study, *S. marcescens* had been implicated as a plant pathogen in only two cases: crown rots of the grain legumes sainfoin (34) and alfalfa (26,40). In both of the latter cases, *S. marcescens* was thought to be part of a pathogen complex, and bacterial species identification was determined without access to current molecular bacterial identification procedures. The fact that *S. marcescens* is also a pathogen of many insect species (14,23,35) suggests the possibility that the interactions between the CYVD bacterium and an insect vector, such as *A. tristis*, may involve more than a simple contamination of legs or mouthparts. In addition, the fact that at least some strains of *S. marcescens* can be opportunistic pathogens of immunocompromised humans prompts interest in the question of whether the CYVD strains differ in this respect from strains derived from other niches.

Phylogenetic trees generated independently from 16S rDNA and *gro*E sequences both show that the two CYVD strains of *S. marcescens*, W01-A and Z01-A, are almost identical to each other. In addition, their branch placement clearly suggests their close relationship to human clinical strains, soil strains, and plant endophytes. However, the CYVD bacteria differ significantly from other strains of *S. marcescens* based on automated substrate utilization assays and fatty acid analysis. The biologically or function-based tests BIOLOG, Vitek, API-20E, and FAME indicate that the metabolic capabilities and fatty acid composition of these cucurbit strains are quite different from those of the type strain of *S. marcescens* as well as from several other reference strains.

In all but one of the cases in which the CYVD strains differed from other strains of *S. marcescens* in the BIOLOG analysis, W01-A and Z01-A were unable to utilize substrates metabolized by the known strains. This interesting observation may reflect niche specificity, the adaptation of the cucurbit strains to a parasitic and pathogenic role within the host plant, in which they no longer require all the proteins or enzyme pathways normally expressed in *S. marcescens*. Interestingly, in fellow *Enterobacteriaceae* member *Escherichia coli*, a span of approximately 130 kbp, starting with the 16S *rrn* gene and ending at the termination of the *groE* gene (a region covering approximately 1/40 of the chromosome), contains at least 10 genes that may be involved in some of the metabolic functions tested in BIOLOG and Vitek and found missing in the CYVD strains: glutamate-aspartate symport protein, putative amino acid/amine transport protein, regulator of melibiose operon, α-galactosidase, melibiose permease II, anaerobic dicarboxylate transport, lysine decarboxylase 1, lysine/cadaverine transport, anaerobic dicarboxylate transport, and aspartate ammonia-lyase (aspartase). If these genes are grouped similarly in *S. marcescens* (a scenario that is not necessarily expected), they could have been lost in a single event.

The fact that known *S. marcescens* strains and those from CYVD-affected plants strongly resemble one another by 16S rDNA and *groE* sequence data, but differ significantly in their metabolic profiles, prompts the question of whether the CYVD bacteria could belong to a different taxonomic group that serendipitously acquired both 16S rDNA and *groE* gene sequences by some mechanism of transformation from *S. marcescens*. However, although *S. marcescens* probably has multiple ribosomal operons (*Enterobacteriaceae* member *E. coli* has seven) (11), our 16S sequence analysis showed no evidence of heterogeneity among putative copies, suggesting that all must be virtually identical. Thus, if one copy had been acquired by transformation from another bacterial species, then all must have been so, an unlikely scenario. To settle this question definitively, entire genome comparisons of numerous strains of CYVD bacteria and known reference strains were performed by repetitive elements-based PCR and by DNA-DNA hybridization analysis; results of those investigations are reported in Zhang et al. (47).

Our findings are similar to those from a recent analysis by 16S rDNA sequencing and DNA-DNA hybridizations of six endophytic bacterial strains isolated from rice roots and stems in the Philippines (17,39). The rice strains were closely related to one another (93% similarity in cluster analysis) and to *S. marcescens* (all within an 86% similarity level). However, as with the CYVD strains, the rice strains differed from the type strain in several key nutrient utilization characteristics, leading the authors to conclude that the rice endophytes represent a novel group of nonpigmented *S. marcescens.*

It is not surprising that long-term association with a particular niche would result in the retention of niche-supportive traits and possible loss of nonessential genes. Signal sensing or regulatory mechanisms operating in the CYVD strains may be responsible for the absence of certain gene products even if the bacteria possess the relevant functional genes. Alternatively, reductive evolution in intracellular bacteria, which may no longer require metabolic functions necessary for a free-living existence, often has led to reduced genome sizes (44). Determination of the genome size of the CYVD strains of *S. marcescens*, work ongoing at this time, will provide insight into possible genome losses.

The wide range of roles played by *S. marcescens* include those of epiphytes, endophytes, soil inhabitants, insect pathogens, and nosocomial human pathogens (1,7,14,16,33,36,38). Our knowledge of the abilities of particular strains of *S. marcescens* to occupy multiple niches, such as being pathogenic to both plants and humans, is only rudimentary. However, the differences in substrate utilization patterns and fatty acid profiles between cucurbit-derived strains of this bacterium and other strains of the species would suggest that substantial changes in gene content or expression, or both, have occurred during the process of this bacterium's adaptation to life in plant sieve tubes.

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